New and Notable

Superresolving Dendritic Spines

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Spines are tiny protrusions that densely stud the dendrites of neurons in the brain. Individual spines are the primary recipients of synaptic inputs from single axons, which emanate from other neurons in the central nervous system. A dendritic tree on one neuron may have hundreds of thousands of spines, making connections to a corresponding number of axons. The morphology of the spine typically consists of an ~1-μm-diameter bulbous head connected to the dendritic shaft by a 50–150-nm-diameter $\times$ 500-nm-long cylindrical neck (Fig. 1). This morphology can be quite variable even on a single dendrite and has been characterized primarily by electron microscopy, because standard light microscopy approaches have insufficient resolution for these tiny structures. But, of course, electron microscopy precludes the analysis of spines in living tissue. To remedy this, a pair of articles in this issue of *Biophysical Journal* (1,2) combines two-photon and stimulated emission depletion (STED) microscopy to characterize individual spine morphologies on neuronal dendrites within freshly harvested live brain tissue (acute brain slices). They increase the resolution of two-photon excitation fluorescence microscopy from ~350 nm to 60–80 nm, which is in the same range as the size of the spine necks (Fig. 1). Determining the size of the spine neck in living tissue will allow neuroscientists to explore how spines can behave as biochemical or electrical compartments to individually process synaptic inputs. Combining the virtues of two-photon microscopy with those of STED holds great promise for unraveling dynamic morphological changes of neuronal spines and their physiological role in the brain.

Typically, in STED microscopy a doughnut-shaped laser beam (nearly zero intensity in the center) is coaligned with the focused excitation beam. The role of this so-called STED beam is to transiently silence all fluorophores in the focal excitation region except those located at the closest proximity of the central zero (3). To this end, the wavelength of the STED beam is tuned toward the red edge of the emission spectrum of the dye so that it instantly quenches excited fluorophore molecules by stimulated emission. If the STED beam is strong enough, it keeps all fluorophores dark, except those located in a tiny, subdiffraction-sized region around the center of the doughnut. Scanning the coaligned beams across the specimen causes features that are just slightly further apart from each other (than the extent of this tiny region) to fluoresce sequentially. Thus, STED microscopy can resolve morphologies that are much finer than the extent of the excitation light spot. Likewise, if the beams are scanned across very fine details, such as spine necks in living brain slices (4–6) or living animal brain (7), the image profile rendered by STED reproduces much better the actual width of the spines (Fig. 1).

Usually fluorescence excitation is accomplished by single photon absorption, that is, with a wavelength in the absorption spectrum of the dye (3). However, in the last few years several groups (5,8–10) have shown that two-photon excitation using the simultaneous absorption of two photons of twice the usual wavelength can be realized in STED microscopy as well, thus pushing the resolution of two-photon microscopy beyond the diffraction barrier. This is highly attractive because two-photon microscopy retains a number of exclusive advantages when imaging relatively thick scattering media such as brain tissue.

Two-photon excitation microscopy is usually performed with a near-infrared train of ~200-fs laser pulses of 12.5-ns temporal distance (80 MHz) as provided by mode-locked Ti:Sapphire lasers. The intense but short pulses of 750–1100-nm wavelength ensure the instant absorption of two photons. In stark contrast, deexciting molecules by stimulated emission requires just a single photon to interact with the (excited) molecule, meaning that STED can be carried out with continuous-wave beams. In fact, the first combinations of STED with two-photon microscopy were realized with continuous-wave STED beams (5,8). This greatly simplified the combination of the two approaches, because the STED beam needed not be synchronized with the ultrafast two-photon excitation pulses arriving every 12.5 ns at the sample; the STED beam is active continuously. However, since the typical lifetime of the excited state is ~2.5 ns, continuous illumination with the STED beam implies that for up to ~80% of the time (~10 out of 12.5 ns) the STED beam is not effective; it just increases the photon load on the sample (11). While this may be fine for thin cell layers, for acute brain slices unnecessary light exposure reduces the number of images that can be taken, due to the onset of phototoxicity. Likewise, since the resolution scales with the square-root of the STED beam power (3) that is really effective, the needless light exposure also curtails the attainable resolution.

To steer clear from these issues, the two groups (1,2) have opted for an experimentally elaborate all-pulsed implementation: each femtosecond

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two-photon excitation pulse is followed by a STED pulse. Both groups use synchronized femtosecond mode-locked Ti:Sapphire lasers as the primary sources for excitation and STED, whereby the STED pulses are stretched out to tens or hundreds of picoseconds. The increased level of sophistication obviously paid off: the attained resolution excels that of standard two-photon microscopy by ~4–6-fold, and up to 20 images of neurons tagged with fluorescent proteins could be recorded without noticeable photodegradation.

The study by Bethge et al. (1) is geared toward imaging green and yellow fluorescent proteins using 592-nm STED pulses from a Ti:Sapphire pumped optic parametric oscillator (OPO). They can take advantage of the large variety of available transgenic rodent strains expressing these commonly used markers as fluorescent labels in particular cell types or subcellular structures. The authors have also implemented two-color STED imaging (12) in acute brain slices, enabling future studies of the interplay between various cell types in the central nervous system, such as neuron-glia interactions. The study by Takasaki et al. (2) uses a dye that is diffused into the neuron. In this case, STED is performed with a Ti:Sapphire laser emitting at ~750 nm. Similarly to the genetically encoded volume label used in the study by Bethge et al. (1), bleached dye molecules are rapidly substituted by new molecules that diffuse into the imaging area. Both studies use long-working-distance water objectives that readily lend themselves to patch-clamping and other electrophysiological recordings. Although these lenses feature a lower numerical aperture than their oil or glycerol immersion counterparts (6), the two studies prove that 60–80-nm resolution is possible deep in living brain slices.

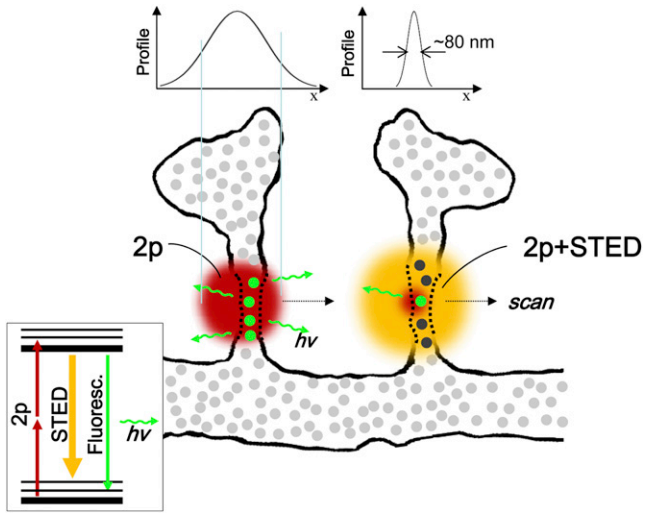

FIGURE 1 Structure and microscopy of dendritic spines. Dendritic spines consist of bulbous heads connected to the dendritic shaft with a thin neck depicted here with a 50–150-nm diameter. (*Left*) An approximate two-photon excitation region (*red*) rendered by a near-infrared focused beam in the specimen plane. (*Right spine*) Doughnut-shaped area (*yellow*) indicates the focal STED beam overlapping the two-photon excitation beam to counteract two-photon excitation throughout the focal region, but not at the doughnut center where fluorescent molecules (*circles*) are still allowed to fluoresce. (*Green*) Fluorescent molecules; (*black*) molecules silenced by STED. When scanning the standard two-photon excitation beam across the spine shaft (*left*), the intensity profile is substantially larger than the actual width of the shaft. In contrast, the combined two-photon STED modality provides improved resolution, rendering a more faithful width of the spine necks upon scanning, as described by the new articles in this issue (1,2).

But why is it important to be able to characterize the morphologies of single spines in live experimental preparations such as brain slices? Because the diffusion barrier produced by the thin spine neck allows the spine head to serve as a biochemical compartment that effectively makes it an independent initial computational element in a neuronal circuit (13). Thus, the dynamics of signaling molecules such as Ca^{2+}, inositol-1,4,5-trisphosphate, or Ca^{2+}/calmodulin-dependent protein kinase II can be different depending on the detailed morphology of an individual spine (14–16). Furthermore, the morphology of an individual spine may itself be dynamic, responding to patterns of synaptic activity (17). This dynamic spine morphology in response to synaptic activity can modulate the postsynaptic signaling dynamics and may be a contributor to mechanisms for learning. Although the biochemical compartmentation afforded by the spine has been studied by many labs with two-photon microscopy for many years, owing to its 4–6-fold improved resolution, the two-photon STED microscopy described by the Sabatini and Nägerl labs (1,2) allows investigators to relate much better synaptic signals to the spine dynamic morphology.

Additionally, there has been accumulating evidence that the thin spine neck may have sufficient electrical resistance so that the spine may also serve as an electrical compartment (18–21). Because depolarization of the spine membrane could produce a positive feedback effect through the opening of voltage-dependent channels, electrical signals within a spine may be even more sensitive to spine morphology than biochemical signals (22). Importantly, two-photon microscopy has recently been used to follow electrical activity in single spines using newly developed voltage-sensitive dyes (23–26).

Progress in laser technology has led to robust and economic fiber lasers providing ready-to-use STED pulses in synchrony with pulses for two-photon excitation. These sources will make the need for Ti:Sapphire/OPO systems in pulsed two-photon STED systems redundant, drastically reducing the level of technical sophistication. For a number of investigations on protein distributions in neurons and spines in particular, the emerging superresolution microscopy method called reversible saturable optical fluorescence transitions (RESOLFT) can be used as an alternative to STED operating at much lower light levels

(27,28). As with most new methods, initial demonstrations usually require complex instrumentation, but once the conditions of operation are understood, massive simplifications follow, making the method readily available.

In summary, uniting two-photon with STED microscopy as shown in the new work published in this issue of *Biophysical Journal* (1,2), offers opportunities to combine detailed morphological characterization with optical electrophysiology to fully explore if and how the dendritic spine can serve as the fundamental computational unit of the mammalian brain.

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